Biol Blood Marrow Transplant 20 (2014) 1290–1300

Biology of Blood and Marrow Transplantation

journal homepage: www.bbmt.org

# Graft-versus-Host Disease Causes Broad Suppression of Hematopoietic Primitive Cells and Blocks Megakaryocyte Differentiation in a Murine Model

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*Article history:*
Received 6 February 2014
Accepted 7 May 2014

*Key Words:*
Hematopoietic stem cells
Hematopoietic progenitor cells
Acute graft-versus-host disease
Cell cycle
Cyclosporine A

A B S T R A C T

Cytopenia and delayed immune reconstitution with acute graft-versus-host disease (aGVHD) indicate a poor prognosis. However, how donor-derived cell hematopoiesis is impaired in aGVHD is not well understood. We addressed this issue by studying the kinetics of hematopoiesis and the functions of hematopoietic stem and progenitor cells in an aGVHD model with haplo-MHC–matched murine bone marrow transplantation. Although hematopoiesis was progressively suppressed during aGVHD, the hematopoietic regenerative potential of donor-derived hematopoietic stem cells remains intact. There was a dramatic reduction in primitive hematopoietic cells and a defect in the ability of these cells to generate common myeloid progenitors (CMPs) and megakaryocyte/erythrocyte progenitors (MEPs). These effects were observed along with a concomitant increase in granulocyte/macrophage progenitors, suggesting that differentiation into MEPs is blocked during aGVHD. Interestingly, cyclosporine A was able to partially reverse the hematopoietic suppression as well as the differentiation blockage of CMPs. These data provide new insights into the pathogenesis of aGVHD and may improve the clinical management of aGVHD.



## INTRODUCTION

In patients with graft-versus-host disease (GVHD), cytopenia is considered one of the most consistent and strongest predictive factors for poor survival [1]. Prolonged thrombocytopenia may require platelet transfusion for the management and prevention of severe bleeding. This condition may also impact treatment outcomes and prognosis [2-5]. However, the exact mechanisms responsible for cytopenia in patients with GVHD are poorly defined.

Hematopoietic stem cells (HSCs) are traditionally defined as cells that are able to produce all types of blood cells in a specific bone marrow (BM) microenvironment. Some key molecules that regulate the self-renewal, proliferation, and differentiation of HSCs during steady-state hematopoiesis have been identified using knock-out mouse models and bone marrow transplantation (BMT)-induced stress [6,7]. However, how the HSC compartment responds to pathological conditions is still poorly understood. In a lipopolysaccharide-induced sepsis model, the expansion of the HSC pool and neutrophil depletion were observed by Rodriguez et al. [8]. The self-renewal ability of HSCs was significantly impaired, which led to insufficient myeloid cell generation to fight sepsis. With chronic infection, Baldridge et al. [9] found that an increased proportion of proliferative long-term repopulating HSCs was caused by IFN-γ. Our previous study showed that the effects of the leukemic environment on HSCs and hematopoietic progenitor cells (HPCs) were distinct in that the repopulation potential of HSCs is preserved, whereas HPCs were exhausted [10]. The results of several mouse studies have shown that the number of donor-derived blood cells is reduced and that Fas-mediated apoptosis is involved in the process [11-14]. However, a systemic investigation of the kinetics of donor-derived hematopoietic stem and progenitor cells (HSPCs) in acute GVHD (aGVHD) hosts is lacking.

In the current study, using a haplo-MHC–matched murine BMT model that recapitulates the BM failure process in hosts with aGVHD, we documented the kinetics of donor-derived HSPCs during aGVHD and explored the underlying mechanisms. Furthermore, based on the current management of aGVHD in the clinic, we used cyclosporine A (CsA) to partially

*Financial disclosure*: See Acknowledgments on page 1299.
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1083-8791/$ – see front matter © 2014 American Society for Blood and Marrow Transplantation.
http://dx.doi.org/10.1016/j.bbmt.2014.05.009

reverse the hematopoietic depression and the differentiation blockage of common myeloid progenitors (CMPs).

## METHODS

### Mice

C57BL/6J (CD45.2$^+$) and BALB/C (CD45.2$^+$) mice were purchased from the Institution of Zoology of the Chinese Scientific Academy (Shanghai, China). B6.SJL-Ptprc$^a$Pepc$^b$/BoyJ mice (B6.SJL, CD45.1$^+$) were obtained from Dr. Tao Cheng (State Key Laboratory of Experimental Hematology, Tianjin, China) and maintained in our animal facility. CB6F1 mice (CD45.2$^+$) were the F1 generation of male C57BL/6J mated to female BALB/C mice. All mice were maintained in a specific pathogen-free condition. All animal protocols were approved by the Institutional Animal Care and Use Committees of all institutions participating in this study.

### BMT Procedures and the Induction of aGVHD

Lethally irradiated (8.0 Gy) female CB6F1 recipients (CD45.2$^+$, 6 to 8 weeks old) were intravenously injected with $5 \times 10^6$ BM nucleated cells (BMNCs) from male B6.SJL mice (CD45.1$^+$, 6 to 8 weeks old) along with $6 \times 10^7$ spleen cells from either female C57BL/6 mice (CD45.2$^+$, 6 to 8 weeks old, the aGVHD group) or female CB6F1 mice (CD45.2$^+$, 6 to 8 weeks old, the control group) 4 to 6 hours after irradiation, as indicated in Figure 1A. We tested various amounts of splenocytes for transplantation (1 to $8 \times 10^7$) and found $6 \times 10^7$ to be the ideal amount for the induction of aGVHD.

The recipient mice were killed every week unless otherwise specified. Hematopoietic reconstitution by the donor-derived cells was monitored. The severity of GVHD was assessed with a clinical GVHD scoring system as first described by Cooke et al. [15]. The following 5 clinical parameters were scored 3 times a week on a scale from 0 to 2: weight loss, posture, activity, fur texture, and skin integrity. The clinical GVHD index was generated by summing the 5 criteria scores (0 to 10). Survival was monitored on a daily basis.

### Flow Cytometry

Mouse BM cells were obtained by flushing ilia, femurs, and tibias as previously described [16]. The immunophenotypes of murine long-term HSCs (LT-HSCs; CD34$^-$Flk2$^-$Lin$^-$c-Kit$^+$Sca-1$^+$, CD34$^-$Flk2$^-$LKS), short-term HSCs (ST-HSCs; CD34$^+$Flk2$^-$Lin$^-$c-Kit$^+$Sca-1$^+$, CD34$^+$Flk2$^-$LKS), multipotential progenitors (MPPs; CD34$^+$Flk2$^+$Lin$^-$c-Kit$^+$Sca-1$^+$, CD34$^+$Flk2$^+$LKS), and HPCs (Lin$^-$c-Kit$^+$Sca-1$^-$, LKS$^-$, including CMPs [CD34$^+$CD16/32$^{lo}$Lin$^-$c-Kit$^+$Sca-1$^-$,CD34$^+$CD16/32$^{lo}$LKS$^-$], common lymphoid progenitors [CLPs; CD34$^+$IL-7$\alpha^+$Lin$^-$c-Kit$^{lo}$Sca-1$^{lo}$], granulocyte/macrophage progenitors [GMPs; CD34$^+$CD16/32$^{hi}$Lin$^-$c-Kit$^+$Sca-1$^-$, CD34$^+$CD16/32$^{hi}$LKS$^-$], and megakaryocyte/erythrocyte progenitors [MEPs; CD34$^-$CD16/32$^{lo}$Lin$^-$c-Kit$^+$Sca-1$^-$, CD34$^-$CD16/32$^{lo}$LKS$^-$]) [17-19] were used to quantify these different cell types within the donor-derived cell populations. All antibodies were purchased from eBioscience (San Diego, CA) unless otherwise noted.

For the detection of donor-derived HSPCs, the following antibodies were used: a mixture of lineage-specific antibodies (biotin anti-CD3, CD4, CD8, B220, Gr-1, Mac-1, and Ter-119), PE-Cy7 anti-Sca-1, PE anti-Flk2 or PE anti-CD16/32, APC anti-c-Kit, PE-Cy5.5 anti-CD45.1, FITCanti-CD34, and streptavidin-conjugated APC-Cy7. For the detection of T cells, B cells, monocytes, and granulocytes, PE anti-CD3, FITC anti-B220, APC anti-Mac-1, PE-Cy7 anti-Gr-1, and PE-Cy5.5 anti-CD45.1 were used.

For HSPCs isolation, c-Kit$^+$ cells were enriched using CD117-conjugated magnetic microbeads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. The enriched cells were stained with antibodies as described above, and then the CD45.1$^+$ CMPs were directly sorted into different tubes and lysed for gene expression analysis. For donor-derived hematopoietic cell (CD45.1$^+$) sorting, BM cells from mice in the aGVHD or control groups were pooled and stained with FITC anti-CD45.1 and PE anti-CD45.2 antibodies (eBioscience, San Diego, CA). A FACS AriaII (BD Biosciences) was used for sorting.

### Cell Cycle and Apoptosis Analyses

In this study, we defined murine HSCs as Lin$^-$Sca1$^+$CD150$^+$cells, MPPs as Lin$^-$Sca1$^+$cells, and HPCs as Lin cells. We did not include c-kit in the immunophenotypes because it is undetectable shortly after BMT [20]. For cell cycle analysis, prestained cells were stained with 10 μg/mL Hoechst-33342 (Sigma Aldrich, St. Louis, MO) and FITC anti-Ki67 (BD Biosciences). The level of apoptosis was measured by labeling BM cells with Annexin-V and the DNA dye 7-aminoactinomycin D (7-AAD, BD Biosciences) in combination with cell surface markers.

### In Vitro Clonal Assay

Two weeks after BMT, CD45.1$^+$ cells were sorted for an in vitro clonal assay and placed in M3434 methylcellulose medium (Stem Cell Technologies, Vancouver, BC, Canada) in 24-well plates with a volume of .5 mL at a density of $2 \times 10^4$ cells/mL with 4 replicate wells. At day 10, the colonies were counted under an inverted microscope and recorded as the number of colonies for the specific lineage.

### Competitive BMT

A competitive BMT model was used as described previously [10,16]. A total of $2 \times 10^6$ sorted CD45.1$^+$ cells along with an equal number of CD45.2$^+$ BMNCs isolated from healthy nontransplanted donor (competitive cells) were cotransplanted into lethally irradiated (8.0 Gy) female CB6F1 recipients ($n = 20$ per group, 6 to 8 weeks old). The chimerism of the peripheral blood (PB) was monitored monthly for up to 6 months. The relative contributions of the donor-derived cells (CD45.1$^+$) and the competitive cells (CD45.2$^+$) to hematopoietic reconstitution were measured by flow cytometry using FITC anti-CD45.1 and PE anti-CD45.2 antibodies.

### Cytokine Profiling

At the early stage after BMT (16 hours and day 3), serum samples were collected from at least 4 mice per group. A Milliplex MAP immunoassay (Merck Millipore, Billerica, MA) was used to determine the cytokine profile according to the manufacturer's instructions. The levels of mouse IL-1α, IL-2, and IFN-γ were examined and reported in units of pg/mL.

### CsA Administration

CsA (Novartis PharmaSchweiz AG, Switzerland) diluted in 200 μL PBS was administered *intraperitoneally* daily to aGVHD mice from day 0 to day 28 at a dose of 10 mg/kg/day.

### Quantitative Reverse Transcriptase PCR

At 2 weeks after BMT, CD45.1$^+$ LKS cells were sorted directly into RNeasy Lysis Buffer (Buffer RLT, Qiagen, Denmark) containing β-mercaptoethanol. Total RNA was extracted with the RNeasy Mini Kit (Qiagen, Denmark). cDNA was synthesized using Improm-II reverse transcriptase (Promega, Madison, WI). Real-time PCR was conducted with SYBR Green Master Mix (Roche, Indianapolis, IN). All the primer sequences are listed in Supplemental Table 1.

### Statistical Analysis

GraphPad Prism 5.0 (San Diego, CA) was used to analyze the results and create graphs. All comparisons represent 2-tailed unpaired *t*-test analyses unless otherwise specified. The flow cytometry data were analyzed with FlowJo Version 7.6.1 software (TreeStar, Costa Mesa, CA).

## RESULTS

### Donor-Derived Hematopoiesis Is Suppressed in aGVHD Hosts

A haplo-MHC–matched BMT mouse model was used in the present study to recapitulate aGVHD after allogeneic (allo)-BMT in humans [21]. CD3$^+$ cells accounted for approximately 30% of transplanted spleen cells (data not shown). All mice in the aGVHD group developed typical aGVHD symptoms and died within 28 days after allo-BMT (Figure 1B). The body weight began to decrease at day 3 in both groups. From day 13 to day 27, the body weight in the aGVHD group was significantly lower than that in the BMT control group (Figure 1C). Histological examinations of the liver, intestines, lungs, and spleen revealed lymphocyte infiltration and inflammation in the aGVHD group but not in the control group (Figure 1D). The average aGVHD score was 2.7 at day 14 after allo-BMT (data not shown). By day 21, all surviving recipients in the aGVHD group developed severe cytopenia (Figure 1E-G), with a 4.4-fold reduction in the WBC count ($P < .0001$), a 1.5-fold reduction in hemoglobin ($P = .0008$), and a 2.3-fold reduction in the platelet count ($P = .0377$) compared with the control group.

To quantify the contribution of donor-derived hematopoietic cells, we monitored the kinetics of the hematopoiesis of donor-derived hematopoietic cells (CD45.1$^+$) in both the PB and BM weekly after BMT. Both the total number and the frequency of CD45.1$^+$ cells in the aGVHD BM were lower than those in the controls (Figure 2A-C). At day 14 after BMT, the total number of BM cells in the aGVHD mice was approximately 44% of that in the control mice. The average

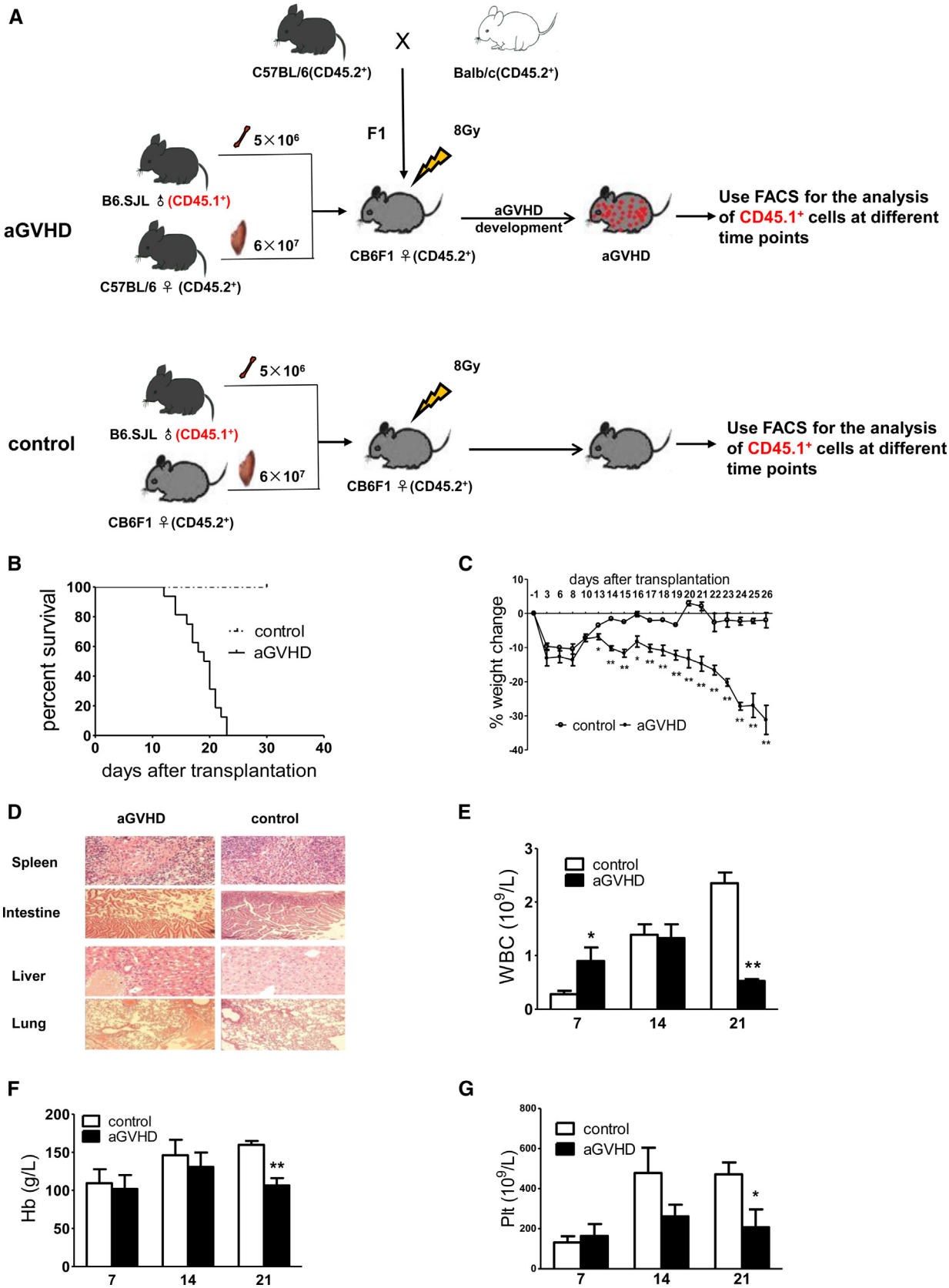
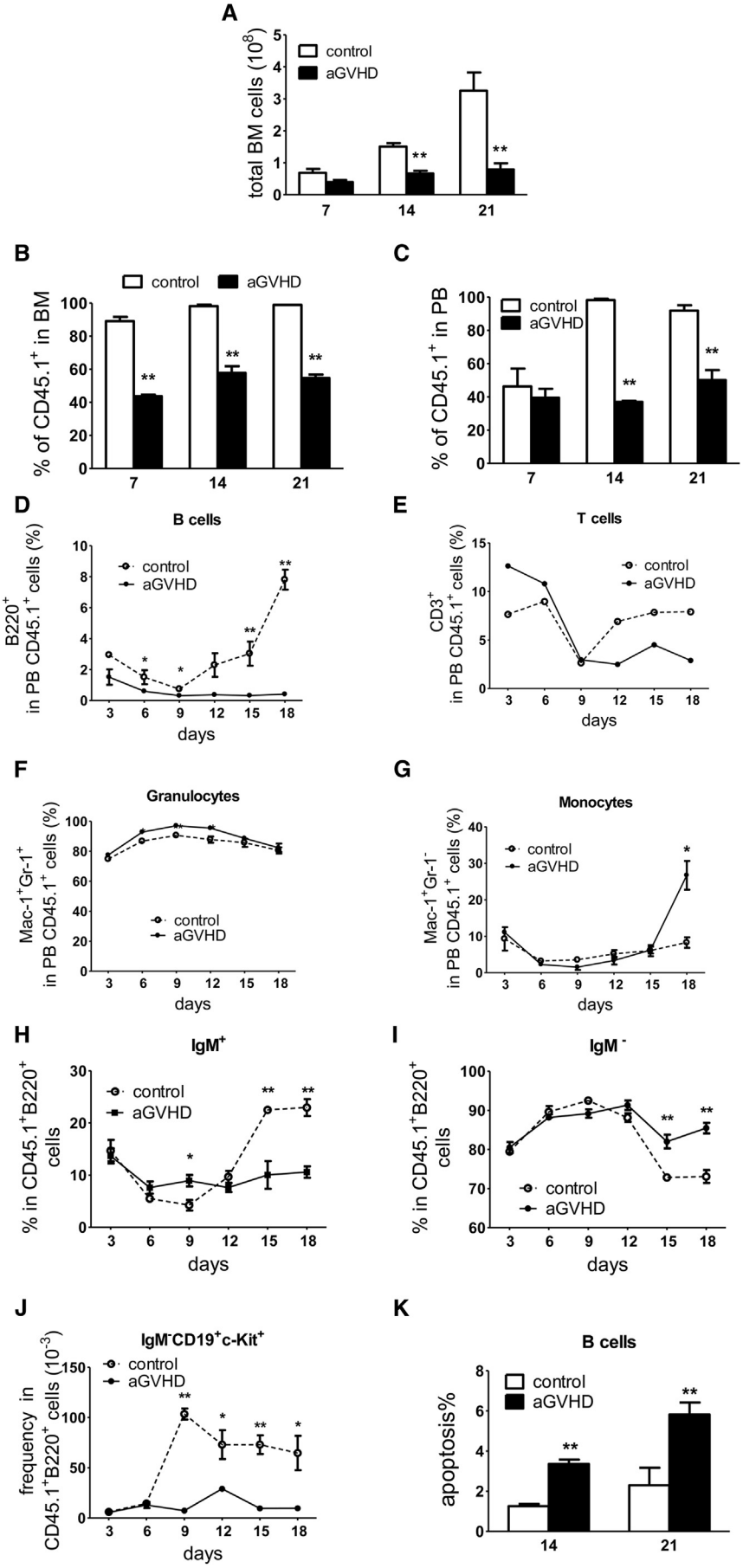



**Figure 1.** Establishment of a murine aGVHD model. A murine haplo-MHC–matched BMT model was established by transplanting $5 \times 10^6$ BMNCs (CD45.1$^+$) from B6.SJL mice into lethally irradiated recipients (8.0 Gy, CB6F1, CD45.2$^+$) along with $6 \times 10^7$ spleen cells from either C57BL/6 mice (CD45.2$^+$, 6-8 weeks old, aGVHD group) or CB6F1 mice (CD45.2$^+$, 6-8 weeks old, the control group) 4-6 hours after irradiation. CD45.1$^+$ donor-derived hematopoiesis was analyzed, and these cells were sorted by flow cytometry at the indicated time points (A). Mice in the control group had significantly longer survival than the mice in the aGVHD group according to the Kaplan-Meier analysis, $P < .0001$, n = 16 in each group (B). (C) Body weights of the 2 groups. (D) Histological changes associated with aGVHD in the spleen, intestines, liver, and lungs (100× magnification). (E) WBC count, (F) hemoglobin level, and (G) platelet count in the PB are shown. Data are expressed as mean ± standard error (SE). $^*P < .05$; $^{**}P < .01$. These results are representative of at least 3 independent experiments and greater than 5 mice in the aGVHD and control groups.


A
total BM cells ($10^8$)
control
aGVHD
B
% of CD45.1$^+$ in BM
C
% of CD45.1$^+$ in PB
D
B cells
B220$^+$ in PB CD45.1$^+$ cells (%)
days
E
T cells
CD3$^+$ in PB CD45.1$^+$ cells (%)
F
Granulocytes
Mac-1$^+$Gr-1$^+$ in PB CD45.1$^+$ cells (%)
G
Monocytes
Mac-1$^+$Gr-1$^-$ in PB CD45.1$^+$ cells (%)
H
IgM$^+$
% in CD45.1$^+$B220$^+$ cells
I
IgM$^-$
J
IgM$^-$CD19$^+$c-Kit$^+$
frequency in CD45.1$^+$B220$^+$ cells ($10^{-3}$)
K
B cells
apoptosis%

percentage of CD45.1$^+$ cells in the aGVHD BM was 57.78% ± 4.10% at day 14 and 54.7% ± 1.71% at day 21 after allo-BMT, significantly lower than the percentages in the control BM, which were stable at approximately 98.95% at day 14 and 91.88% at day 21, respectively ($P < .05$). A similar trend was observed in the PB.

B lymphopenia in aGVHD has been reported in several clinical and experimental studies [22-27]. The evaluation of mature subsets also showed that B lymphopoiesis was particularly affected. At day 18 after BMT, the percentage of B lymphocytes was 0.41% ± .15% in the aGVHD group versus 7.81% ± .65% in the control group ($P = .0004$, Figure 2D). The frequency of T cells was lower in aGVHD group when compared with control mice, but without statistical difference (Figure 2E). Furthermore, the frequencies of granulocytes and monocytes were close to or even higher than those in the control mice at the later stage of aGVHD development. Those cell lineages even exhibited signs of recovery in the presence of aGVHD (Figure 2F-G). These data are consistent with those in a previous report [28]. Further experiments revealed that the differentiation of the B cell lineage was blocked at the stage of pro-B cells (CD19$^+$c-Kit$^+$IgM$^-$, Figure 2H-J). The evaluation of apoptosis within the CD45.1$^+$B220$^+$ population showed a higher percentage of Annexin V–positive cells in aGVHD mice at day 14 and day 21 after allo-BMT (Figure 2K). However, the level of B cell apoptosis (3% to 6%) in the aGVHD mice suggested that apoptosis only partially accounted for the reduction in B lymphocytes in the aGVHD hosts. Collectively, these data demonstrate that in the context of BM suppression by aGVHD, B lymphopoiesis was particularly affected, whereas no significant changes were observed in the frequencies of myeloid cells.

### *Hematopoietic Suppression Occurs at the Stem and Progenitor Cell Levels, but the Hematopoietic Regenerative Potential of Donor-Derived HSCs Remains Intact in aGVHD Hosts*

To determine whether the suppression of donor-derived hematopoietic cells (CD45.1$^+$) in the aGVHD BM was due to a negative impact on HSPCs, we quantified the frequencies and absolute numbers of HSCs and HPCs (Figure 3A,B). At day 14, absolute numbers of LKS, LKS$^-$, and Lin$^-$ cells were lower in the mice with aGVHD than in the controls. Although the total numbers and frequencies of donor-derived cells (CD45.1$^+$ cells) in the aGVHD BM were significantly lower than those in the control BM, a higher frequency of LKS cells, a cell subset greatly enriched for HSPCs, was observed among donor-derived hematopoietic cells. The frequencies of LT-HSCs, ST-HSCs, and MPPs were therefore increased (Figure 3C,D). The input (CD45.1$^+$CD34$^-$Flk2$^-$LKS) and output (CD45.1$^+$ BMNCs and CD45.1$^+$ LKS) cells harvested at day 14 were measured. The yield of LKS cells per initial CD34$^-$Flk2$^-$LKS cell in the aGVHD hosts was 1.5 times lower than that in the controls (Supplemental Table 2). An independent quantitation of HSCs using the signaling lymphocytic activation molecule markers further confirmed the significant inhibition of HSPC regeneration in the aGVHD hosts (Supplemental Figure 1A-D).

HSCs progressively differentiate into progenitor cells, which, in turn, generate various mature blood cell lineages. Our results showed that the frequencies of CMPs and CLPs were decreased in mice with aGVHD (Figure 3E), and the frequency of MEPs was also significantly lower in the aGVHD BM than in the control BM, whereas the frequency of GMP was increased (Figure 3F,G). The increased GMP frequency was consistent with the increased frequencies of the Mac-1$^+$Gr-1$^+$ and Mac-1$^+$Gr-1$^-$ populations in PB, as described above (Figure 2F,G). The suppression of CLPs during aGVHD may contribute to the barely detectable level of B lymphocytes in the PB (Figure 2D).

At day 14 after BMT, CD45.1$^+$ cells from the aGVHD and control groups were harvested for an in vitro clonal functional assay. The donor-derived CD45.1$^+$ cells from the aGVHD mice had a higher capacity to generate colonies from the committed progenitors (Figure 4A). Together with the phenotypic analysis, these data provide functional evidence for the increased frequencies of HPCs as measured by the CFC (colony-forming cell) assay during aGVHD development.

We next examined the repopulating ability of hematopoietic cells from aGVHD hosts in a new host by competitive transplantation. Engraftment analysis based on PB chimerism (CD45.1 versus CD45.2) was performed monthly after transplantation. The level of CD45.1$^+$ cells from the aGVHD hosts increased 1 month after cBMT. Six months after cBMT, the engraftment of CD45.1$^+$ cells from the aGVHD hosts was 2 times greater than that from the control group (19.37% versus 8.89%; $P = .0171$; Figure 4B). Of note, the frequency of LT-HSCs in $2 \times 10^6$ hematopoietic cells harvested from aGVHD hosts was almost 2 times higher than that in cells harvested from the control group (Figure 3C). These data indicate that there was no apparent defect in the repopulation potential of donor-derived HSCs from the aGVHD environment. A multilineage analysis of PB from cBMT recipients revealed that the HSCs in the aGVHD environment maintained their ability to differentiate into both myeloid and lymphoid lineages at a level comparable with that of the control group (Figure 4C).

### *Donor-Derived Stem/Progenitor Cells in the aGVHD Hosts Tend to Be More Quiescent without a Homing Defect*

To address the mechanism that caused the absolute number of donor-derived (CD45.1$^+$) cells to be significantly decreased in the aGVHD BM, we examined whether this reduction is related to the insufficient homing of donor-derived cells or changes in cell proliferation or apoptosis. We first evaluated the homing rate by examining the percentage of donor-derived (CD45.1$^+$) cells in the BM 16 hours after BMT as previously described with minor modifications [29]. There were no significant differences in the homing efficiency between the 2 groups (Figure 5A). We further quantified the homed stem/progenitor cells, and the frequencies of primitive cells among CD45.1$^+$ cells were not

**Figure 2.** Suppression of donor-derived hematopoietic regeneration during aGVHD development. Total BM cellularity and the percentage of donor-derived hematopoietic cells (CD45.1$^+$) in both BM and PB were monitored from day 7 to day 21 after BMT (A, B, and C). The frequencies of B cells (B220$^+$), T cells (CD3$^+$), granulocytes (Mac-1$^+$Gr-1$^+$), and monocytes (Mac-1$^+$Gr-1$^-$) in the PB were measured every 3 days after BMT (D, E, F, and G, respectively). After BMT, BMNCs were harvested every 3 days to evaluate the frequencies of immature and mature B cells (B220$^+$IgM$^+$) (H), B-cell precursors (B220$^+$IgM$^-$) (I), and a class of pro-B cells (B220$^+$CD19$^+$c-Kit$^+$IgM$^-$) (J). The results are shown as mean ± SE. $^*P < .05$; $^{**}P < .01$ (n = 3 for each time point, *t*-test). The data shown are from 1 of 3 experiments with similar results. The proportion of apoptotic CD45.1$^+$B cells (Annexin V$^+$/7-AAD$^-$) in aGVHD BM or control BM was measured at day 14 and day 21 after BMT (K). The results are shown as mean ± SE. $^*P < .05$; $^{**}P < .01$ (n = 6-16 per each group, *t*-test). Data shown are representative of 4 experiments with similar results.

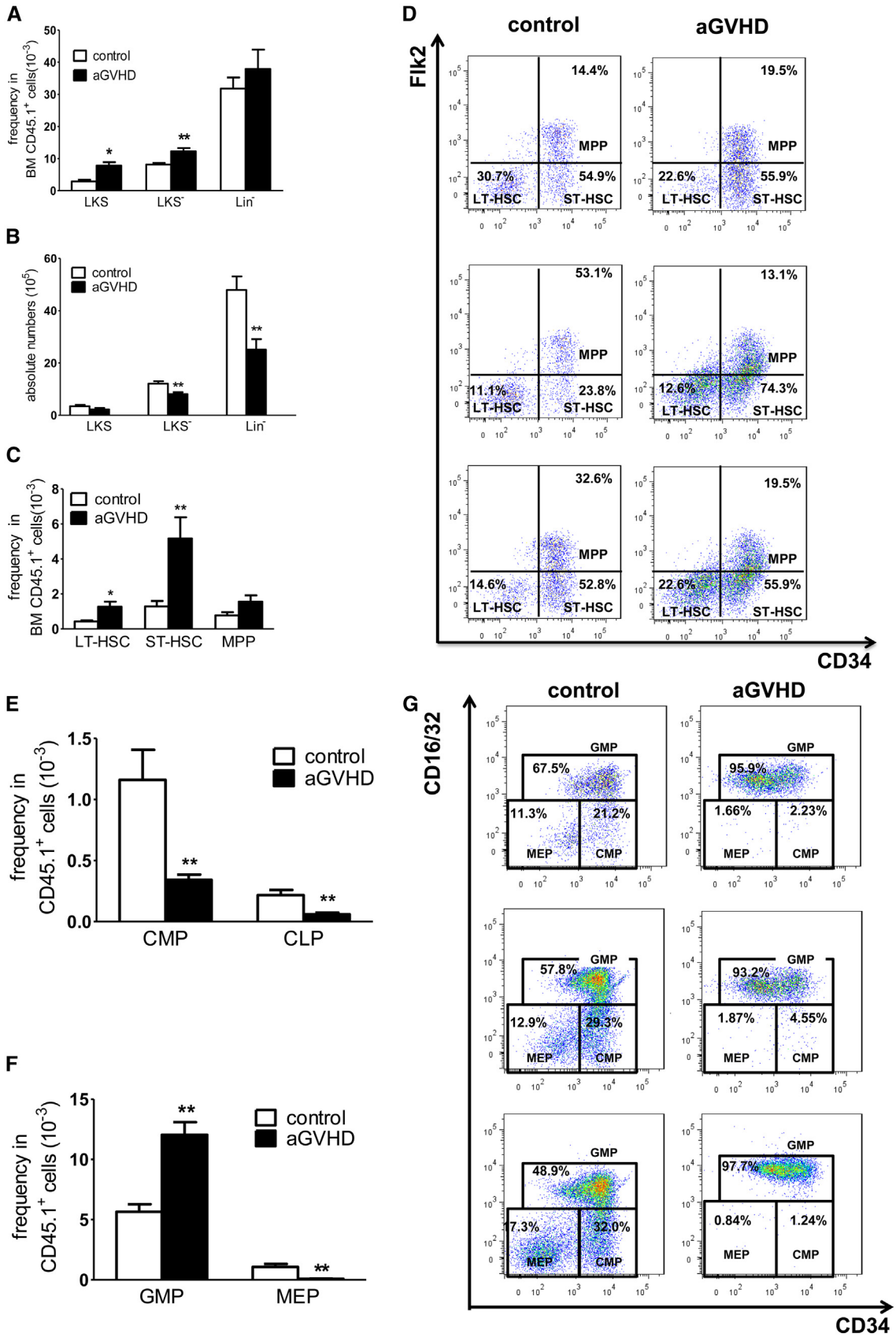



**Figure 3.** Kinetics of the hematopoiesis of donor-derived HSCs/HPCs in aGVHD mice. The frequencies and absolute numbers of LKS, LKS$^-$, and Lin$^-$ cells among CD45.1$^+$BMNCs at day 14 after BMT (A and B). Flow cytometric analysis of LT-HSCs, ST-HSCs, and MPPs among CD45.1$^+$ BMNCs at day 14 after BMT (C). Representative data for LT-HSCs, ST-HSCs, and MPPs among the CD45.1$^+$ BMNCs from aGVHD or control BM at day 14 after BMT (D). The frequencies of more-committed progenitors, including CMPs, CLPs, GMPs, and MEPs, in the donor-derived BMNCs (CD45.1$^+$) (E and F). Representative data for CMPs, GMPs, and MEPs among the CD45.1$^+$ BMNCs from aGVHD or control BM at day 14 after BMT (G). The results are shown as mean ± SE. $^*P < .05$; $^{**}P < .01$ (n = 7-21 per each group, *t*-test). The data shown are representative of 3 experiments with similar results.

significantly different between the 2 groups (Supplemental Figure 2).

It is well known that quiescence is associated with the quality of stem cells [16,30]. The quiescent fraction of donor-derived primitive cells in the cell cycle was examined using costaining with anti-Ki67 and the DNA dye Hoechst 33342. A significantly larger fraction of $CD45.1^+Lin^-Sca1^+CD150^+$cells from the aGVHD BM were in $G_0$ phase, suggesting the aGVHD BM is able to keep the HSCs in a more quiescent state (Figure 5B, Supplemental Figure 3). Interestingly, the percentage of apoptotic HSCs was almost equivalent between the aGVHD BM and the control BM when examined by Annexin V and 7-AAD staining (7.9% ± 1.92% versus 6.65% ± 1.06%, $P = .66$), and there was no significant difference in the apoptosis rates of HPC and MPP subsets as well (Figure 5C), indicating apoptosis may not account for the decrease in primitive hematopoietic cells in the aGVHD BM. Thus, our results demonstrate that HSCs were kept in a more quiescent state, and neither an altered homing process nor apoptosis contributed to the suppressed hematopoiesis in the aGVHD hosts.

### *MEP Differentiation Is Blocked in the aGVHD Hosts*

We showed dramatic reductions in CMPs and MEPs in terms of both frequency and absolute number, yet there was a concomitant increase in GMPs (Figure 3E,F). Further analysis showed that the ratio of MEPs to CMPs was significantly lower among the aGVHD $CD45.1^+$ BM cells than among the control BM cells, suggesting that a blockage may exist in cell differentiation toward MEPs (Figure 6A). To elucidate the mechanism, we first examined the levels of apoptosis of $CD45.1^+$ MEPs in the experimental and control groups but found no significant difference (Figure 6B).

On day 14 after BMT, we collected $CD45.1^+$ CMPs and analyzed them with quantitative reverse transcriptase PCR. The expression levels of transcriptional factors essential for MEP differentiation [31], including *c-MPL* (CD110), *RUNX1*, and *CCND1*, were significantly decreased in CMPs isolated from the aGVHD hosts compared with the levels in CMPs isolated from the controls. Quantitative reverse transcriptase PCR analysis of *c-MPL*, a negative regulator of MEP differentiation, showed that its expression level was 1.5-fold lower in the $CD45.1^+$ CMPs, if not LT-HSC, from the aGVHD BM than in the cells from the control BM (Figure 6C,D). These results suggest that the differentiation blockage may occur at the CMP level. The differential expression of these transcription factors may contribute to the underlying molecular basis of the blockage of the differentiation of CMPs into MEPs.

### *CsA Attenuates the Hematopoietic Suppression and Differentiation Blockage in aGVHD Hosts*

We found that the serum levels of Th1 cytokines, including IL-1α, IL-2, and IFN-γ, were significantly higher in the aGVHD mice than in control mice (Supplemental Figure 4). Because elevated Th1 cytokine levels are a key indicator of donor T lymphocyte proliferation and differentiation, we investigated whether inhibiting T cell immunity could attenuate the hematopoietic suppression associated with aGVHD. It has been reported that CsA can inhibit the activity of allo-reactive $CD8^+$ T cells [32] and that human graft-versus-host reactions can be effectively prevented by the administration of CsA [33-36] We thus administered CsA to the aGVHD mice to determine whether it can rescue the BM suppression or restore the quantity of donor-derived HSCs.

After the daily injection of CsA (10 mg/kg) from day 0 to day 28 (Figure 7A), all mice in the CsA group remained

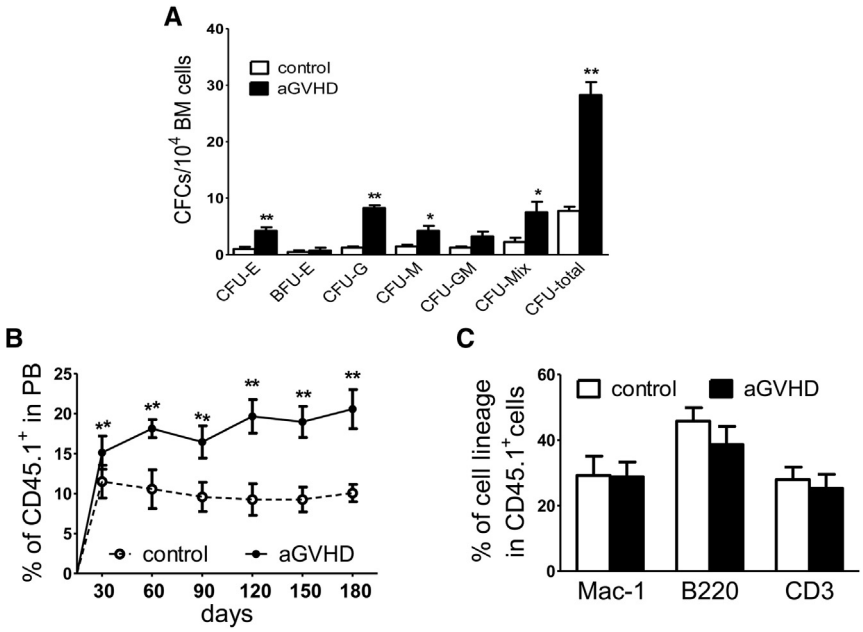



**Figure 4.** In vitro colony assay and in vivo cBMT of donor-derived ($CD45.1^+$) cells. Two weeks after BMT, mice were killed and BM harvested. The donor-derived hematopoietic cells ($CD45.1^+$) were sorted for in vitro colony assays. For the CFC assay, the sorted $CD45.1^+$ cells were cultured in defined methylcellulose medium supplemented with a cytokine cocktail. CFU-E, BFU-E, CFU-G, CFU-M, CFU-GM, and CFU-Mix represent CFU-erythrocyte, BFU-erythrocyte, CFC-granulocyte, CFC-monocyte, CFC-granulocyte and monocyte, and CFC-mix (more than 2 lineages), respectively (A). The results are shown as mean ± SE. $^*P < .05$; $^{**}P < .01$ (n = 4 per each group, *t*-test). The hematopoietic regeneration of donor-derived hematopoietic cells ($CD45.1^+$) in aGVHD or control mice was examined in secondary recipients using the cBMT assay, in which equal numbers of test ($CD45.1^+$) and competitor cells ($CD45.2^+$) were cotransplanted into lethally irradiated CB6F1 ($CD45.2^+$). The overall reconstitution levels of donor-derived HSCs in the primary recipients were monitored for 6 months after cBMT (B). Multilineage differentiation of the engrafted cells was analyzed 6 months after cBMT (C). Mac-1, B220, and CD3 were used as markers of myeloid ($Mac\text{-}1^+$), B ($B220^+$) and T ($CD3^+$) cells, respectively. The results are shown as mean ± SE. $^{**}P < .01$ (n = 3-5 per each group, *t*-test). The data shown are representative of 3 experiments with similar results.

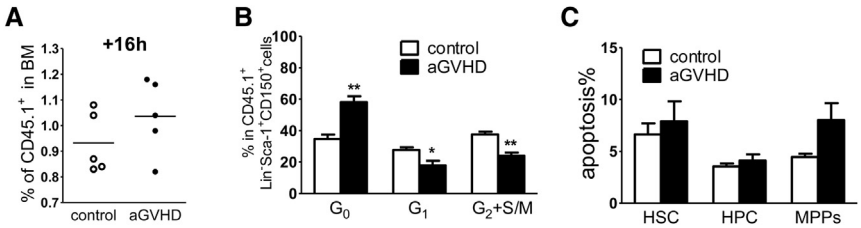



**Figure 5.** Homing, apoptosis, and cell cycle of primitive hematopoietic cells in aGVHD BM. As illustrated in Figure 1A, $5 \times 10^6$ BMNCs from B6.SJL mice were transplanted into lethally irradiated recipients along with $6 \times 10^7$ spleen cells from C57BL/6 mice or CB6F1 mice. (A) In vivo homing assay. At the 16-hour time point, BMNCs were harvested and stained with CD45.1-FITC and CD45.2-PE. The homing efficiency was calculated with the equation $CD45.1^+/(CD45.1^+ + CD45.2^+)$. %. The results are shown as mean ± SE. $P > .05$ (n = 5 per each group, *t*-test). (B) At day 14 after BMT, BMNCs were harvested and stained with anti-Ki67 and Hoechst 33342. The quantification of the $G_2$ + S/M phase and $G_0/G_1$phase cell percentages in the $CD45.1^+Lin^-Sca1^+CD150^+$ subpopulation indicated more quiescent $CD45.1^+Lin^-Sca1^+CD150^+$ cells in aGVHD animals. The results were obtained from 3 independent experiments. Each value is mean ± SE. $^*P < .05$; $^{**}P < .01$ (n = 5-8 per each group, *t*-test). (C) Percentages of apoptotic (Annexin $V^+$/7-$AAD^-$) cells among the $CD45.1^+$ HSCs ($Lin^-Sca\text{-}1^+CD150^+$), MPP ($Lin^-Sca\text{-}1^+$) and HPC ($Lin^-$) subsets at day 14 after BMT are shown. Results are shown as mean ± SE. All $P > .05$ (n = 3-5 per each group, *t*-test). Data shown are representative of 3 experiments with similar results.

healthy and active 5 weeks after BMT, whereas all mice in the aGVHD group became overtly ill at day 14 after allo-BMT and died within 4 weeks from exhaustion. Furthermore, daily injections of CsA prevented the decrease in BM cellularity (Supplemental Figure 5). The BM cellularity was increased by 2.0-fold, and B lymphopoiesis was also rescued with decreased apoptosis after CsA administration (Figure 7B).

The populations of LKS and $LKS^-$ cells were also recovered. At day 14 after CsA administration, the absolute numbers of LKS and $LKS^-$ cells in the CsA-treated mice were 1.97-fold and 1.51-fold higher than those in the aGVHD group, respectively (Figure 7C). Further analysis of the MEP and CMP subsets revealed that the frequency of MEPs was increased by 5-fold, 0.1‰ versus 0.5‰, $P < .001$ (Figure 7D,E), by CsA administration, and this increase was accompanied by an increase in the ratio of MEPs to CMPs from .24 to .42, $P = .0074$ (Figure 7F), indicating that the administration of CsA can partially reverse the blockage of MEP differentiation in mice with aGVHD.

## DISCUSSION

Previous studies have shown that BM suppression occurs in addition to alterations in the microenvironment during aGVHD pathogenesis [28,37]. Although these studies clearly demonstrated the occurrence of myelosuppression during aGVHD, they were limited to the analysis of terminally

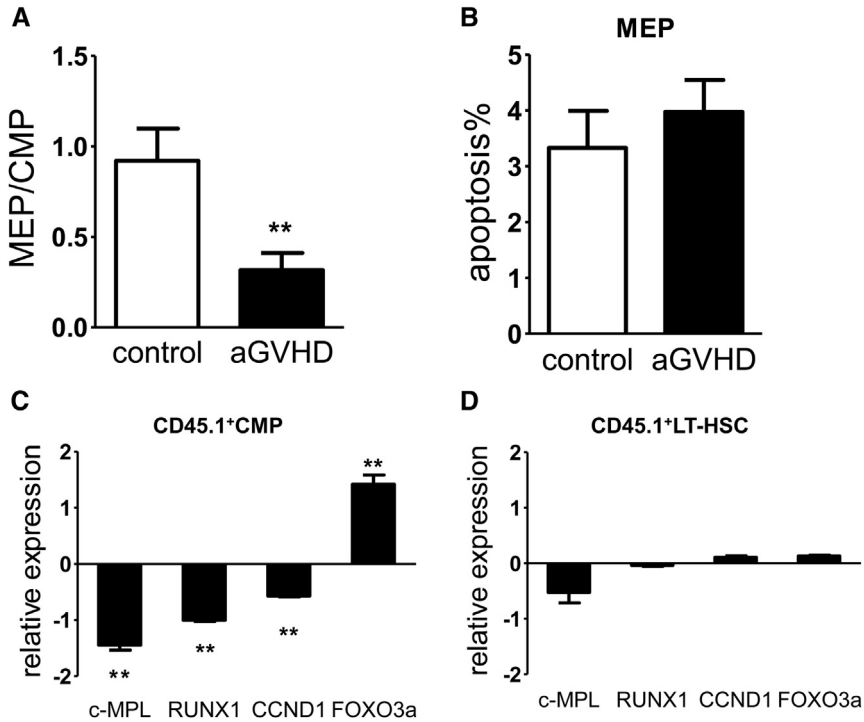



**Figure 6.** Blockage of differentiation in the hematopoietic hierarchy in aGVHD hosts. (A) The ratio of MEPs to CMPs within the $CD45.1^+$ population was measured at day 14 after BMT, $^{**}P = .0015$ (n = 10 per each group, *t*-test). (B) The percentage of apoptotic (Annexin $V^+$/7-$AAD^-$) cells among the $CD45.1^+$ MEP subset was measured at day 14 after BMT. $P = .5924$ (n = 3, control; n = 11, aGVHD; *t*-test). (C and D) A sample of RNA was extracted from sorted $CD45.1^+$ CMPs or $CD45.1^+$ LT-HSCs and analyzed for the expression of *c-MPL*, *RUNX1*, *CCND1*, and *FOXO3a* by quantitative reverse transcriptase PCR. The bar graphs present the averages of the log values of the fold change in expression for 3 independent samples in the aGVHD group compared with those of control mice. Results are shown as mean ± SE. $CD45.1^+$ CMPs: *c-MPL*, $^*P = .0127$; *RUNX1*, $^{**}P = .0001$; *CCND1*, $^{**}P = .0009$; *FOXO3a*, $^*P = .0329$; $CD45.1^+$ LT-HSCs: *c-MPL*, $P = .0602$; *RUNX1*, $P = .2565$; *CCND1*, $P = .0713$; *FOXO3a*, $P = .1096$ (n = 3 per each group, *t*-test). The data shown are representative of 3 experiments with similar results.

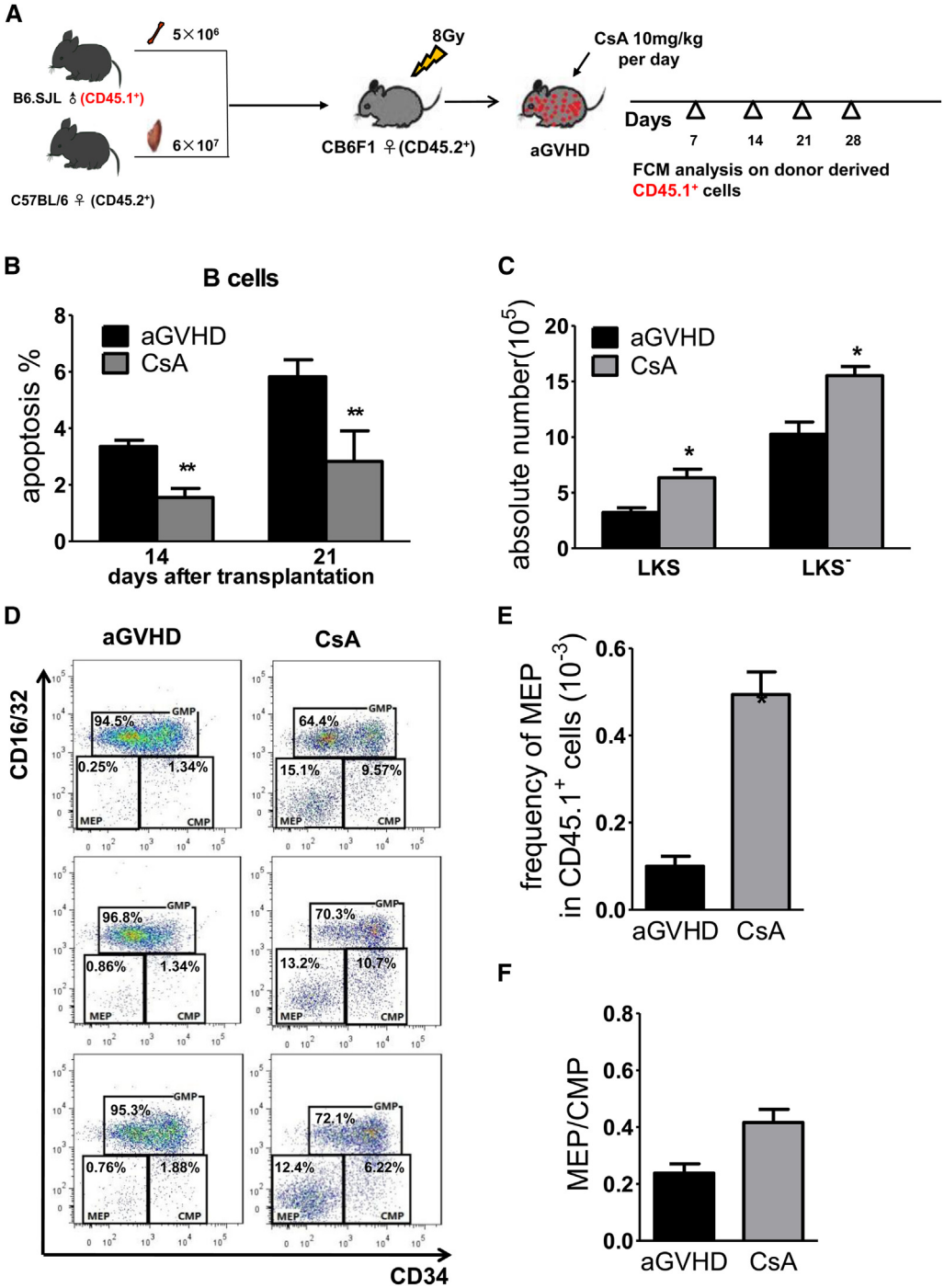



**Figure 7.** Attenuation of the hematopoietic suppression and differentiation blockage in aGVHD hosts by CsA. The haplo-MHC-matched aGVHD model was established as above (Figure 1A). The mice in the CsA group received CsA at 10 mg/kg/d from day 0 to day 28 intraperitoneally. Then, the mice were killed at the indicated time points, and the donor-derived CD45.1$^+$ cells in the BM were analyzed (A). The percentage of apoptotic CD45.1$^+$ B cells (Annexin V$^+$/7AAD$^-$) was measured at day 14 and day 21 after BMT in mice treated with or without CsA (B). $^{**}P < .01$ (n = 3 per each group, *t*-test). The absolute numbers of LKS cells and LKS$^-$ cells among the CD45.1$^+$ BMNCs in the 2 groups were measured at day 14 after BMT as shown in the graph (C). LKS: $^*P = .024$, LKS$^-$:$^*P = .0191$ (n = 3 per each group, *t*-test). (D) Representative flow cytometric analysis of CMPs, GMPs, and MEPs in the donor-derived CD45.1$^+$ population at day 14 after BMT with or without CsA. The frequencies of MEPs in CD45.1$^+$ cells and the ratios of MEPs to CMPs at day 14 after BMT in aGVHD mice with or without CsA are shown in the graphs (E and F). Frequencies of MEPs: $^*P < .001$ (n = 8 per each group, *t*-test); MEP/CMP: $P = .0074$ (n = 8 per each group, *t*-test). The data shown are representative of 4 experiments with similar results.

differentiated blood cells. The kinetics of HSPCs involved in this process was not documented in detail. Our data indicate that the cytopenia observed in our model can be largely attributed to the suppression of HSPCs and defective differentiation into certain lineages, such as B cells and MEPs, although the altered microenvironment and other factors may also contribute to this process. HSCs were kept quiescent in the presence of aGVHD stress but did not have an altered homing ability. Although the absolute number of HSCs was decreased, their self-renewal ability remained intact.

Our previous work showed that normal HSCs recovered from T leukemic hosts were still highly functional after being seeded into a nonleukemic host [10]. The similarity of the performance of HSCs in these 2 studies is due to the arrest of the cell cycle under stress [10]. Indeed, cell cycle arrest is a protective mechanism and a common cellular response to stress [8,10,38]. Therefore, when more-quiescent HSCs from aGVHD hosts are seeded into a new host, these cells reenter the cell cycle, and the self-renewal potential of the HSCs can be fully restored (Figure 4B).

Our study thus establishes a link between HSPCs and thrombocytopenia in aGVHD, a previously unappreciated connection. Prolonged thrombocytopenia is a frequent complication after allo-HSC transplantation. A low platelet count on the 60th day after transplantation has been shown to be an independent risk factor for poor prognosis in allo-HSC transplantation patients [39]. Other studies have linked poor prognoses to thrombocytopenia 100 days after allo-BMT [4,40]. The major causes of thrombocytopenia include accelerated peripheral platelet destruction by antiplatelet antibodies and the insufficient production of platelets from marrow megakaryocytes; the latter mechanism may play a predominant role [41,42]. In the present study, we found that during aGVHD, the CMP subset was significantly decreased and there was a blockage in differentiation to MEPs. Consequently, GMPs were increased and correlated with the augmented levels of granulocytes and monocytes in the PB (Figure 2F,G). Changes to the CMP subset were associated with cell cycle arrest and growth suppression of the HSC pool. These results are further supported by the in vitro functional characterization of HSPCs (Figure 4A).

Consistent with the finding of decreased MEPs in aGVHD BM, CMPs from aGVHD mice had significant lower expression levels of *c-MPL,* an important regulator of the development of megakaryocytes and platelets (Figure 6C,D). C-Mpl (CD110), which is expressed on the surfaces of megakaryocytes and megakaryocyte precursors, is a receptor for thrombopoietin (TPO). Yamazaki et al. [42] found that plasma TPO was significantly increased in HSC transplant recipients with thrombocytopenia in comparison with those without; however, no study has focused on c-Mpl (CD110) expression in megakaryocytes in allo-HSC transplantation context. Although we did not measure TPO status in aGVHD hosts, we found the primitive hematopoietic subset was unresponsive to TPO, resulting in significantly fewer CFU megakaryocytes derived from $CD45.1^+$ cells isolated from aGVHD BM in vitro, which aggravated the thrombocytopenia (data not shown).

The secretion of the inflammatory cytokines IL-1$\alpha$, IL-2, and IFN-$\gamma$ is closely related with expansion of donor $CD8^+$ T cells in the early stage of aGVHD, as shown in previous studies [21,43]. Evolving inflammatory cytokines disrupt the BM structure, which may interfere with the physiological interactions between HSCs and their niches. Interestingly, when the aGVHD hosts were treated with CsA, the frequencies and function of HSPCs were partially restored. These effects could be primarily due to the suppressive effect of CsA on the expansion of allo-reactive $CD8^+$ T cells. A previous study by Shono et al. [28] showed that the impaired BM stroma and osteoblasts cannot support hematopoiesis in aGVHD hosts, and the present study provides the immune cues for the hematopoietic cues. Therefore, the actual effects of the aGVHD environment on normal tissue stem cells are most likely the result of multiple factors, including the model and stage of aGVHD, the conditioning regimen, and even the defined phenotype of primitive hematopoietic cells. Our model underscores the importance of the immune environment or conditions in dictating the specific functional state of tissue stem cells. Because CsA can only partially restore the BM cellularity in aGVHD hosts, it is possible that other factors may contribute to the inhibition of HSPCs and MEP differentiation.

Taken together, our study demonstrates for the first time that the pathological condition of aGVHD may negatively affect HSPCs. The resulting hematopoietic suppression, especially the blocked differentiation or commitment along B cells or megakaryopoietic/erythropoietic lineage, may ultimately cause cytopenia. Interestingly, these abnormalities can be partially improved by the administration of CsA, an effective agent for managing aGVHD in patients. However, caution must be taken before these findings can be generalized and applied to humans, especially in the situation where MHC-matched donors are used. Our current findings might be more relevant to the haplo-BMT for patients.

## ACKNOWLEDGMENTS

*Financial disclosure:* Supported by the National Natural Science Foundation of China (81090413, 81090411, 81270638, 81270567, 30871100, 30800488, and 81300416), the Ministry of Science & Technology of China (2011CB964800, 2012CB966600, and 2010CB945204), and the Shanghai Science and Technology Committee (08JC1406500 to J.W.). T.C. was a recipient of the Scholar Award from the Leukemia & Lymphoma Society (1027-08) and an Outstanding Young Scholar Award from the National Natural Science Foundation of China (30825017). X.H. was sponsored by a scholarship from the Shanghai Health Bureau (XYQ2011007).).

*Conflict of interest statement*: There are no conflicts of interest to report.

*Authorship statement*: Y.L. and X.H. contributed equally to this work.

## SUPPLEMENTAL DATA

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.bbmt.2014.05.009.

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